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Article Body Template

A perspective on targeting inflammation and cytokine actions in atherosclerosis

- **Abstract:**

Atherosclerosis, a chronic inflammatory disorder of the vasculature that results in cardiovascular disease, continues to pose a significant health and economic burden on modern society. Whilst inflammation has generally been accepted as the key driver of all stages in the disease, it was not until recently that inhibition of a specific pro-inflammatory cytokine (interleukin-1β) yielded successful results in the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial. This article offers a perspective on targeting inflammation for atherosclerosis, focusing on results of recent phase 3 clinical trials, and discusses other potential candidates together with future challenges and prospects.

- **Keywords:** Atherosclerosis; Inflammation; Cytokines; Cardiovascular disease; Interleukin-1β

- **Main body of text:**

1. Introduction

Cardiovascular disease (CVD) is currently responsible for more than a quarter of global mortalities, with around 7.4 million people living with the condition in the UK alone (according to British Heart Foundation statistics (2019)). The rising worldwide prevalence is attributed to increasing diabetes and obesity, coinciding with widespread adaptation of a 'westernized' lifestyle, and continues to pose a significant health and socioeconomic burden on modern society. The prevailing challenge of reversing the consequences of increasingly high-risk, sedentary lifestyles and maintaining cardiovascular health has been a fundamental incentive for much of previous and current research, as well as healthcare initiatives. CVD, which includes myocardial infarction (MI), coronary heart disease (CHD), angina pectoris and cerebrovascular accidents (stroke), is often the end consequence of atherosclerosis; a chronic inflammatory disorder of the vasculature. For over 50 years, atherosclerosis has remained the top cause of morbidity and mortality in Western civilization and is currently the number one cause of deaths globally [1]. Despite clinical advancements over the last few decades that have positively influenced patient prognosis, the significant and persisting residual risk of recurrent adverse events has fueled the need for alternative approaches in cardiovascular medicine.

2. Atherogenesis and disease progression

Atherosclerosis is characterized by the formation of fibrous plaques within the walls of medium and large arteries. Sites of bifurcations and inner curvatures, which have been shown to be associated with persistent low-grade inflammation [2], are particularly susceptible to atherosclerosis initiation. Atherosclerotic plaques, or atheromas, are derived mainly from cholesterol deposition and accumulation within the subendothelial space, resulting in enhanced

occlusion and compromised cardiovascular function (illustrated in Figure 1 and described in more depth by [3-5]). Key factors of the disease, hyperlipidemia, oxidative stress and inflammation, are inextricably linked and collectively orchestrate pro-atherogenic processes that contribute to the increasingly dysfunctional phenotype. Endothelial dysfunction is the key initiating step of atherogenesis, with plaque development involving the formation of lipid-rich foam cells within the arterial intima and vascular smooth muscle cell (SMC; VSMC) proliferation and migration (see Figure 1). Elevated circulating low-density lipoprotein (LDL) cholesterol (LDL-C) is a key risk factor of atherogenesis, as these particles may infiltrate and accumulate within the vessel wall, where, locally-produced reactive oxygen species (ROS) induce their modification into oxidized LDL (oxLDL) [6]. Therefore, LDL-C has widely been used as a major marker and predictor of CVD risk [7].

Previously, it was widely believed that LDL infiltrates the endothelial barrier via passive diffusion; however, recent advancements suggest that scavenger receptor (SR) class B type 1 (SR-B1) facilitates the delivery of LDL into the artery wall [8]. The oxLDL then triggers an immune response and activation of the surrounding endothelial cells (ECs). This results in the recruitment and migration of circulating monocytes and T-lymphocytes to the site of LDL accumulation. The oxLDL may also encourage necroptosis (a recently discovered, non-apoptotic programmed cell death pathway) which induces morphologic changes to cells similar to that of necrosis, and inflammation within the surrounding tissues [9]. The recruited monocytes differentiate into macrophages under the influence of macrophage-colony stimulating factor (M-CSF) and excessively take up oxLDL, which is facilitated by SRs (e.g. CD36 and SR-A1) [10], to become lipid-laden foam cells; a hallmark of atherosclerosis (dysregulated lipid homeostasis in atherosclerosis is described in detail by [4]). Macrophages possess a high level of heterogeneity and plasticity, and are recognized to be fundamental instigators and drivers of atherosclerosis [11]. Although macrophages are beneficial and necessary for clearing the accumulated LDL initially, these cells can undergo metabolic reprogramming encouraged by different stresses within the atherosclerotic microenvironment [11]. This includes macrophage proliferation [12] and the production of inflammatory mediators that enhances pro-inflammatory/atherogenic processes. The resulting macrophage inflammation drives lesion formation and atherosclerosis development. Whilst macrophages were previously thought to be main precursors of foam cells in atherosclerotic lesions, advances in lineage-tracing techniques have recently shown VSMCs to also be a key cell type present in all stages of atherosclerosis, with the ability to adopt a range of phenotypes that resembles foam cells, macrophages and others [1]. VSMCs are therefore now also regarded as key contributors of foam cell formation [13], possessing a greater phenotypic plasticity than was previously acknowledged [1].

Death of the foam cells via apoptosis, necrosis and necroptosis (which stimulates additional infiltration of monocytes) [9], leads to the accumulation of cholesterol, apoptotic cells and cellular debris, leading to the formation of a lipid-rich necrotic core. The growth of this necrotic core, furthered by continued cell death and defective efferocytosis, exacerbates the

inflammatory burden with the release of a plethora of pro-inflammatory cytokines. Plaque progression involves the formation of a fibrous cap that encloses the plaque, and coincides with the infiltration of additional VSMCs from the tunica media to the intima, as they undergo phenotypic shift from a quiescent to a synthetic state (reviewed extensively by [1]). In the later stages, this plaque-stabilizing fibrous cap is broken down by the activity of matrix metalloproteinases (MMPs) (produced mainly by lesional macrophages), resulting in plaque destabilization and subsequent rupture. Vulnerable plaques tend to consist of a large lipid-rich necrotic core with a thin, unstable and highly-inflamed fibrous cap abundant in monocytes, macrophages and T-lymphocytes [10]. The consequence of plaque rupture is thrombosis that, depending on the anatomical location of the thrombus within the vascular bed, can manifest as cerebrovascular disease, peripheral vascular disease, or coronary artery disease, to name a few [1].

3. Current pharmacological interventions

3.1 Statins: Blockbuster drugs with issues

The primary intervention for atherosclerosis and CVD patients is lifestyle modifications to increase physical activity and reduce dietary lipid consumption, combined with therapeutic strategies that interfere with cholesterol metabolism to counteract hyperlipidemia. Currently, standard pharmacological agents mainly aim to reduce circulating LDL-C levels. Statins, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, remain the first choice, cornerstone pharmaceutical intervention for the prevention of primary and secondary major adverse cardiovascular events (MACE). Statins reduce endogenous cholesterol synthesis [14] and increase hepatocyte LDL receptor (LDLR) expression to reduce plasma LDL-C [10]. Although statins reportedly produce a 22% risk reduction in cardiovascular events for every mmol/L reduction in LDL-C [15-17] and hence are effective and successful drugs, many patients on statin therapy fail to achieve target cholesterol levels despite maximal dosage [18-20]. Other issues of statin therapy include tolerability and adverse side effects [21], contributing to non-compliance and subsequent uncontrolled LDL-C levels and residual risk. Clinical trials have identified significant residual cardiovascular risk in statin-treated patients, despite successful lowering of plasma cholesterol levels [22-24]. This is despite the additional anti-inflammatory and other pleiotropic actions of statins [25]. Therefore, it is often difficult to gauge whether the protective actions of statins are due to its LDL-C-lowering ability, anti-inflammatory activity, or a combination of both.

3.2 Other strategies targeting LDL-C

Other lipoprotein-lowering interventions include monoclonal antibodies targeting proprotein convertase subtilisin/kexin type-9 (PCSK9), evolocumab and alirocumab, which prevent PCSK9-mediated degradation of the LDLR and thereby encourage liver LDL-C absorption [26]. Both antibodies have been shown to reduce LDL-C by over 50% in two phase 3 clinical

trials [27]. Key issues are, however, their high expense and questionable cost-effectiveness as regular subcutaneous injection is required. Thus, despite demonstrating significant CVD benefit in the large FOURIER trial (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Patients With Elevated Risk) involving 27,564 patients [28], the cost restricts their application in clinical practice. Other strategies for PCSK9 inhibition include gene silencing via small interfering RNA (siRNA) to target the translation stage of PCSK9 synthesis [29]. Inclisiran has demonstrated promising LDL-C-lowering ability in phase 1 and 2 clinical trials, and the on-going ORION program will assess its effect on cardiovascular outcomes in approximately 15,000 atherosclerotic CVD patients in a phase 3 trial [29]. Another option is ezetimibe, which reduces LDL-C by inhibiting the absorption of cholesterol at the intestinal brush border [30]. Clinical studies have reported a 15-22% reduction in LDL-C after ezetimibe monotherapy [10] and results from clinical trials [20, 31] also support its use as a co-therapy with statins.

Despite some promising outcomes as discussed above, the significant residual risk for CVD in statin-treated patients emphasizes the detrimental effects of unresolved, chronic inflammation. Whilst statins have considerably decreased CVD-related mortalities over the years, currently-standard interventions are inadequate for completely combating this residual risk of recurrent events in post-MI patients [20, 32, 33], emphasizing the significant role of inflammation independent of hyperlipidemia and the need for therapeutic avenues beyond lipoprotein homeostasis. Furthermore, the anti-inflammatory actions of statins could be a major contributor to its ability to reduce CVD. Indeed, results of the JUPITER trial (Justification for the Use of Statin in Prevention: An Intervention Trial Evaluating Rosuvastatin) showed rosuvastatin to significantly reduce the incidence of primary cardiovascular events in those with systemic inflammation without elevated LDL-C levels (and so would not have qualified for statin therapy) [34]. The promising results provided the rationale for further clinical trials investigating the efficacy of other anti-inflammatory therapies (discussed later in Section 5). Since MI and strokes can also occur in patients without hyperlipidemia [35], the capability of ongoing inflammation to negatively-affect patient outcome despite successful lowering of LDL-C is highlighted. Importantly, increased levels of high-sensitivity C-reactive protein (hsCRP) (a non-specific biomarker of inflammatory status) have been identified in patients on statin therapy [36, 37], indicating that the anti-inflammatory actions of statins are inadequate for completely combating residual inflammation and subsequent CVD risk in these patients.

4. Inflammation in atherosclerosis

Both pro-inflammatory and anti-inflammatory cytokines have roles in modulating cardiovascular health. In atherosclerosis, the former prevail, tipping the balance in favor of inflammation rather than its resolution. Cytokines are a large group of proteins subdivided into several classes that include interleukins (ILs), chemokines, tumor necrosis factors (TNFs) and interferons (IFNs), which orchestrate inflammation and modulate both the innate and adaptive immune responses. Pro-inflammatory cytokines are fundamental drivers of atherosclerosis; their activities can enhance the inflammatory state, modulate foam cell formation, and

influence plaque development and progression through to clinical sequelae arising from its rupture [3, 6, 38]. It is therefore unsurprising that cytokines have long been recognized as potential therapeutic targets for atherosclerosis. Various pro-inflammatory cytokines are expressed in atherosclerotic plaques, and all cell types implicated in the disease are able to produce, as well as respond to, these cytokines. However, the exact mechanisms underlying how pro-inflammatory cytokines contribute to plaque destabilization remains elusive, although it is generally accepted that increasing inflammation positively correlates with risk of plaque rupture. Key pro-atherogenic cytokines, such as IL-1β, IFN-γ and TNF-α, are implicated in multiple processes that propagate atherosclerotic plaque development, as shown in Figure 1 and previously dealt with in our reviews [3, 38] (latter published in this journal). Therefore, given the fundamental role of inflammation in atherosclerosis, dampening inflammatory processes via suppression of pro-inflammatory cytokines may naturally attenuate disease progression and reduce plaque burden. Many pre-clinical studies blocking the actions of pro-atherogenic cytokines, such as IL-1β, most-commonly using neutralizing antibodies (or less-commonly, using soluble decoy receptors) have highlighted their therapeutic potential.

4.1 Inflammasome activation

IL-1β is a particularly potent pro-atherogenic cytokine capable of inducing macrophage polarization to the M1, pro-inflammatory phenotype, as well as being a major activator of innate immunity and capable of inducing self-expression [39, 40]. IL-1β stimulates the production of IL-6 (by various cell types) which promotes the synthesis of acute phase reactants (e.g. fibrinogen and plasminogen activator inhibitor) and CRP by hepatocytes [41]. CRP is hence a marker of elevated inflammation and potentially a better predictor of MACE in comparison to LDL-C [42]. Production and release of IL-1β can be stimulated by multiple factors. LDL modification and foam cell lysis can stimulate the production of 'danger signals' which activate pattern recognition receptors, including SRs, toll-like receptors (TLRs) and NOD-like receptors (NLRs) by macrophages and other cells of the innate immune system [43]. Uptake of cholesterol crystals by macrophages via macropinocytosis and crystallization of intracellular cholesterol activates the NLR family, pyrin domain containing 3 (NLRP3) inflammasome [44]. This process involves lysosomal destabilization, ROS release and the action of the caspase-1 protease, resulting in the cleavage and secretion of potent pro-inflammatory cytokines, IL-1β and IL-18 [44, 45]. This inflammasome is hence a key driver of IL-1β-mediated inflammatory responses and can also be activated by other 'danger signals', including calcium phosphate crystals and oxLDL within macrophages [45]. Whilst deficiency in NLRP3 or caspase-1 failed to inhibit macrophage infiltration and atherosclerosis progression [46], deficiency in IL-1β has been found to reduce disease severity [47], and monoclonal antibodies targeting IL-1β successfully inhibited plaque formation [48] in apolipoprotein E deficient (*ApoE*$^{-/-}$) mice. Therefore, these *in vivo* data support the crucial involvement of IL-1β, in atherogenesis and disease progression, demonstrating that its suppression attenuates plaque development.

5. From bench to bedside: clinical trials exploiting anti-inflammatory therapies

5.1 The CANTOS trial and CIRT

Whilst pre-clinical studies have confirmed the immense potential of targeting cytokines/inflammation in atherosclerosis, large clinical trials have only been carried out recently. In particular, the outcomes of two randomized, double blind, placebo-controlled clinical trials have opened up insights into the application of anti-inflammatory therapies for the prevention of atherosclerosis-related events (despite one of these being unsuccessful). Results of the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial found that administration of the human IL-1β monoclonal antibody, canakinumab, to previous MI patients with elevated hsCRP, significantly reduced the occurrence of MACE by 17% (at 150 and 300 mg doses) without lowering plasma cholesterol levels [49] (summarized in Table 1). In contrast to this, the use of a broad spectrum anti-inflammatory, low-dose methotrexate, in the Cardiovascular Inflammation Reduction Trial (CIRT) had no effect on IL-1β, -6 or hsCRP and subsequent incidence of cardiovascular events [50] (see Table 1). This is despite the association between low-dose methotrexate use and fewer cardiovascular events in arthritic patients identified in previous observational studies [51-53]. However, the neutral results from the CIRT also echo those obtained from other clinical trials that use other broad-spectrum anti-inflammatory agents (mentioned later in Section 5.2).

Table 1. Comparison of the two phase 3 clinical trials, CANTOS and CIRT.

	CANTOS	CIRT
N	10,061	4786
Inclusion criteria	Previous MI and hsCRP level of ≥2 mg/L	Previous MI/multivessel coronary disease and type 2 diabetes/metabolic syndrome
Intervention	Canakinumab	Low-dose methotrexate
Dose(s)	50, 150 and 300 mg Subcutaneous injection every 3 months	Target dose of 15-20 mg weekly
Biological basis	To target the IL-1β/6 pathway of inflammation	Broad-spectrum anti-inflammatory
Results	150 and 300 mg reduced hsCRP by ~35-40% with no significant reduction in LDL-C after 48 months IL-6 was reduced to similar extent when measured at 12 months	No reduction in IL-1β, -6 or hsCRP
Outcome	Significantly lowered rate of recurrent cardiovascular events	No observed benefits

The CANTOS trial was the first large clinical trial to yield positive results with the use of an IL-1β neutralizing antibody, which successfully reduced the incidence of secondary MACE

compared to placebo. The promising results yielded from this trial not only demonstrate the possibility and promise of targeting key cytokines implicated in atherosclerosis to improve outcomes, but also provides solid proof of the inflammation hypothesis. However, it is also necessary to explore why the CIRT was less successful to better guide the development of anti-inflammatory strategies in the future. It is important to mention the two key differences in the patient cohorts between the two trials; whilst hsCRP levels ≥2 mg/L was a mandatory inclusion criterion in the CANTOS trial, this was not the case for the CIRT. Therefore, the lack of effect by low-dose methotrexate could be attributed to the absence of elevated (or less severe) systemic inflammation due to amplified IL-1β signaling in this patient cohort. This is supported by the disparity between the median baseline CRP levels (and IL-6 to a lesser extent) between the two patient populations, which were much higher in the CANTOS cohort compared to that of the CIRT, and so the CIRT cohort did not necessarily represent residual inflammatory risk like that of the CANTOS. Also noteworthy is that although the actions of low-dose methotrexate are thought to suppress the actions of proinflammatory cytokines (IL-2, -6 and TNF-α), stimulate anti-inflammatory cytokine production, and dampen macrophage activation and the T-helper-1 response [54], it has no specific effects on the IL-1β/6 pathway, thereby explaining the lack of reduction in hsCRP levels after treatment. Furthermore, perhaps implications of co-morbidities, which is common in CVD patients, and other parameters affecting cardiovascular health should also be given careful consideration when predicting response to treatment. The CIRT cohort consisted of patients with previous MI or multivessel coronary disease combined with type 2 diabetes or metabolic syndrome, and a higher percentage of patients with hypertension than that of the CANTOS trial, and so other factors may have had an impact on treatment success. It would therefore have been interesting to directly compare the effect of canakinumab to low-dose methotrexate on previous patients of MI with elevated hsCRP levels to verify this and further confirm the effectiveness of targeting IL-1β in at-risk patients.

In summary, results of both trials suggest that targeting a specific pro-atherogenic cytokine is a more effective approach than targeting inflammation using broad-spectrum agents that perhaps have a smaller effect on a number of pro-inflammatory mediators, rather than a targeted effect on one. Whilst inflammation has long been accepted to underpin the pathophysiology of atherosclerotic disease, the CANTOS trial effectively translated years of scientific research into reality, demonstrating that targeted reduction of IL-1β levels successfully reduces inflammatory burden and subsequent occurrence of secondary cardiovascular events in human patients. Taken together, these data largely support the effectiveness of using CRP as a biomarker of inflammation and the potential of targeting the IL-1/6 pathway to attenuate residual inflammatory risk in statin-treated patients and the incidence of adverse cardiovascular events. In contrast to this, a recent study by Gomez et al. [55] using smooth muscle cells (SMC) lineage tracing in *ApoE*$^{-/-}$ mice found IL-1β to encourage plaque stability in late-stage atherosclerosis. Results of this study showed that treatment with IL-1β antibody reduced SMC and collagen content, but increased macrophages within the fibrous cap, with no change in lesion size itself and full inhibition of beneficial outward

remodeling. Therefore, IL-1β may not be solely pro-atherogenic with effects depending on the stage of disease, stressing the complex etiology and pathophysiology of atherosclerosis, which may, in part at least, be responsible for the clinical challenge of preventing primary and secondary MACE.

5.2 Other trials

Other major large phase 3 clinical trials that exploit anti-inflammatory therapies for atherosclerotic disease include the LoDoCo (Low-dose colchicine for secondary prevention of CVD) [56], SOLID-TIMI (Effect of darapladib on major coronary events after an acute coronary syndrome) [57] and LATITUDE-TIMI (Effect of losmapimod on cardiovascular outcomes in patients hospitalized with acute MI) [58] trials. Of these, benefit was only observed in the LoDoCo trial, which used colchicine, a broad-spectrum anti-inflammatory previously employed to treat inflammatory disorders such as gout and recurrent pericarditis [56]. In contrast to low-dose methotrexate, colchicine is an alkaloid whose anti-inflammatory properties include antitubulin activity that inhibits neutrophil function [59], which may therefore exert its positive, anti-atherogenic effects by inhibiting neutrophilia and subsequent neutrophil-mediated secondary infiltration of monocytes into the lesion [60]. In a cohort of 532 patients with stable coronary disease receiving aspirin and/or clopidogel and statins, treatment with 0.5 mg/day of colchicine significantly reduced prevalence of cardiovascular events (4.5%) compared to placebo (16.0%) though a small percentage of patients showed intestinal intolerance towards colchicine [61]. These promising results suggest a potential for even targeting key leukocytes implicated in inflammatory processes that underpin atherosclerosis and directly contribute to lesion growth. There are also a number of other clinical trials (mainly phase 3) investigating the ability of colchicine to prevent adverse cardiovascular events associated with coronary disease (e.g. LoDoCo2 [62], COACS (Colchicine for Acute Coronary Syndromes; NCT01906749)) and cerebrovascular disease (e.g. CONVINVE (Colchicine for Prevention of Vascular Inflammation in Non-cardio Embolic Stroke; NCT02898610)), with more results due soon. Results of the very-recent trial COLCOT (Colchicine Cardiovascular Outcomes Trial), involving 4,745 post-MI patients (≤30 days), showed that 0.5 mg/day of colchicine significantly reduced the risk of ischemic cardiovascular events compared to placebo, with benefits in relation to cardiovascular end points comparable to those of canakinumab in the CANTOS trial [61]. In a small patient subgroup (where data were available), CRP was reduced by over 65% in the first 6 months post-MI with similar changes in leukocyte counts observed in both groups (although not significant) [61]. However, these observations should be interpreted with caution due to the small size of the subgroup that was not randomly selected from the full cohort and hence offer limited representation. Furthermore, a much longer follow-up period with a larger patient cohort and quantitative assessment of CVD and inflammation biomarkers is required to ascertain the safety and efficacy of long-term colchicine treatment.

Other studies have explored the use of IL-1 receptor antagonist (IL-1ra) therapy on cardiovascular outcomes, such as the MRC-ILA Heart Study (Effect of IL-1ra on markers of inflammation in non-ST elevation acute coronary syndromes) [63] and the VCU-ART trial

(Virginia Commonwealth University Anakinra Remodeling Trial) [64-66]. The MRC-ILA study is a phase 2 clinical trial involving 182 patients with non-ST elevation acute coronary syndrome (ACS; NSTE-ACS) presenting <48 hours from the onset of chest pain. This study investigated the effects of short-term IL-1ra administration on hsCRP levels post-NSTE-ACS, since IL-1 drives CRP levels during NSTE-ACS. Daily subcutaneous IL-1ra injection at 100 mg/day for 14 days significantly lowered hsCRP and IL-6 levels below those of day 1, and these were also significantly lower when compared to placebo [63]. There was also significant suppression of leukocyte count throughout the treatment (without overt neutropaenia). However, there was no significant difference in MACE at 30 days or 3 months between the treatment and the placebo groups. This is unsurprising since cessation of IL-1ra treatment was associated with an increase in hsCRP and IL-6 levels, suggesting 14 days treatment is insufficient for continued suppression or controlling post NSTE-ACS inflammation. However, power limitations of this study prevent conclusive analysis of clinical outcomes and so the overall effect on IL-1ra treatment on CVD risk cannot be inferred from these results. Further studies with larger cohorts over a longer term are required to determine the optimal treatment duration, safety and efficacy of IL-1ra for ACS patients.

The VCU-ART [67] and VCU-ART2 [65] similarly investigated the effect of 100 mg/day anakinra, a recombinant IL-1ra, for 14 days on adverse cardiac remodeling in post-acute MI (AMI) patients. When results of both these studies are combined (N=40), anakinra significantly dampened elevations of CRP levels, and hence acute inflammation associated with ST-segment elevation AMI (STEMI), during the first 72 hours compared to placebo. However, about half of those in the anakinra group experienced an increase in CRP compared to baseline, and importantly, CRP levels remained heightened beyond the upper limit of normal, suggesting residual cardiovascular risk. This dose of anakinra, or anakinra monotherapy, may hence be insufficient to combat the acute and severe inflammation that occurs with STEMI. Re-evaluation of the dose and duration, as well as combining with another anti-cytokine/inflammatory therapy may be necessary. Whilst the overall incidence of new heart failure was lower in the treatment group with an absolute risk reduction of 25% at 3 months follow-up, additional larger clinical trials are required to determine the short and long-term safety and efficacy of anakinra treatment for the prevention of adverse cardiac remodeling and secondary cardiovascular events. Key limitations include the small sample size, even after combining the VCU-ART and VCU-ART2 cohorts, as well as the lack of true baseline measurements which restrict assessment of treatment efficacy and accurate quantitation of its effect on biomarkers of the IL-1-mediated inflammatory response. Investigators also admit to overestimation of the treatment effect on left ventricle remodeling in the first VCU-ART study, resulting in too optimistic a sample size in the VCU-ART2, and so neither studies possessed sufficient power to detect differences in specific clinical events. These studies suggest anakinra has promise for combating the amplified IL-1-mediated inflammatory response in STEMI but progression onto a phase 2 clinical trial involving 99 patients (results to be published) [66] will provide more solid evidence.

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly and widely used anti-inflammatory therapies, but cardiovascular toxicity associated with their use (especially high dose, long-term) has been a cause for concern. NSAIDs induce analgesic and anti-

inflammatory effects via inhibition of cyclooxygenase (COX), which exists in two isoforms; COX-1 (constitutively expressed in majority of the cells) and COX-2 (induced by proinflammatory stimuli) [68]. Meta-analyses analyzing NSAIDs and coxibs, selective COX-2 inhibitors (developed to combat gastrointestinal side-effects associated with COX-1 inhibitors) in 31 [69, 70] and 280 [71] trials have correlated these, especially coxibs, with increased risk of adverse cardiovascular events. As the degree of risk appears dose and duration-dependent, the use of minimum effective dose and duration, and avoidance in high-risk CVD patients is hence recommended.

Taken together, the phase 2 and 3 clinical trials detailed above illustrate that specific targeting of IL-1 is a promising avenue and vital in controlling inflammation and subsequent residual cardiovascular risk.

6. The future of anti-inflammatory therapies

As we enter and progress through the era of streamlined medicine to tailor treatments for individual patients, the use of accurate biomarkers is proving increasingly important for implementation of more effective treatment strategies. The CANTOS trial also provided evidence supporting the use of CRP quantitation to assess inflammatory burden and risk of MACE [49]. How best to translate this to medical practice will likely become a major focus of future research; identification of relatively cheap and readily available inhibitors of IL-1β is necessary, given the prevalence of atherosclerotic disease and its clinical consequences in modern society. Whilst canakinumab has proven effective in combating residual inflammation within an at-risk patient cohort, the predominant dilemma is its estimated low cost-effectiveness, if applied as a standard secondary MACE prevention treatment strategy based on current prices [72], which are even greater than that of PCSK9 inhibitors. This will no doubt heavily restrict its use as a last resort to the most high-risk patients only after exhaustion of other therapeutic options. Therefore, the persisting need for alternative avenues and cheaper agents is ever-more essential.

6.1 Other potential targets for anti-cytokine therapy

Although targeting IL-1β has proven successful in down-regulating inflammation and subsequent occurrence of recurrent cardiovascular events, there is still room for improvement to further reduce residual inflammatory and cardiovascular risk, warranting exploration of other potential target cytokines. Given the promise of targeting the IL-1/6 pathway, it would be logical to explore other closely associated cytokines. Production of IL-6 by various cell types is stimulated by IL-1β, and so a small amount of IL-1β can induce the generation of a large level of IL-6, which in turn induces liver CRP production [73]. Therefore, targeting the IL-1/6 pathway further downstream may also elicit similar beneficial effects to inhibiting IL-1β. In previous *in vivo* studies, inhibition of IL-6 *trans*-signaling using soluble glycoprotein 130 has been shown to attenuate atherosclerosis by inhibiting endothelial dysfunction, VSMC infiltration and monocyte recruitment [74]. Increasing IL-6 via injection of the cytokine also exacerbated atherosclerotic lesion size [75, 76], and IL-6 lentivirus is capable of inducing plaque destabilization [77] in $ApoE^{-/-}$ mice. Surprisingly, IL-6 deficiency in both major animal

models of atherosclerosis (*ApoE*$^{-/-}$ and *LDLr*$^{-/-}$) has been shown to promote atherosclerosis [3], suggesting pleiotropic effects that may not be strictly pro-atherogenic, perhaps highlighting the often-ambiguous roles different cytokines play depending on the microenvironment. Despite the same also being true for IL-1β, the reduction in prevalence of secondary MACE, as shown by results of the CANTOS trial, suggest that whilst completely removing select pro-inflammatory cytokines may not positively impact disease progression and may exacerbate it, lowering its levels acquires benefit.

IL-18 is another pro-inflammatory cytokine cleaved and secreted by activation of the NLRP3 inflammasome alongside IL-1β [44] and mediates the actions of IFN-γ, which is capable of inducing the activation of pro-inflammatory, M1 macrophages, increasing foam cell formation and inducing foam cell apoptosis [38, 78], all processes capable of exacerbating inflammatory burden and inciting plaque destabilization. In previous *in vivo* studies, IL-18 deficiency decreased atherosclerosis in *ApoE*$^{-/-}$ mice, which was associated with reduced IFN-γ activity and increased plaque stability [79, 80]. Moreover, IFN-γ deficiency has been found to eliminate the detrimental effects of IL-18 on atherosclerosis, and so targeting IFN-γ may inhibit pro-atherogenic effects of both cytokines. Therefore, these studies have facilitated tremendous advancement in our knowledge of the underlying mechanisms of atherosclerosis and along with large scale clinical trials, should continue in the quest for alternative therapeutic strategies.

6.2 Challenges

The CANTOS trial offered the first evidence that targeting a specific pro-inflammatory cytokine can successfully prevent recurrent cardiovascular events. The results emphasize not only the importance of select pro-inflammatory cytokines in driving disease progression and onset of clinical manifestations, but the vitality of comprehensive assessment of patient suitability for anti-cytokine therapy, due to the overriding concern of compromising immune response and increasing infection risk. Cohorts excluded from the CANTOS trial included immunocompromised patients and those suffering from chronic or recurrent infection, highlighting the concern of further-diminishing immune function in already-susceptible individuals that inevitably accompanies any strategy that targets or manipulates mediators of the immune system. Although there was no difference in all-cause mortality, canakinumab was in fact associated with a higher incidence of fatal infection (caused by gram positive bacteria) compared to placebo [49], and so may need to be restricted to high-risk patients. Additionally, in the COLCOT, a higher proportion of patients in the colchicine group experienced diarrhea and nausea, with infection and pneumonia also being more common in the colchicine group compared to placebo, suggesting a change in immunologic responses [61]. Key issues of concern continue to revolve around applying anti-cytokine therapy to immunocompromised patients and the conundrum of risk vs benefit. Furthermore, the other smaller, phase 2 trials emphasize the need for individualized approaches to match treatment dose and duration with inflammation intensity and disease severity [65]. This is especially imperative due to the different sources and complexity of proinflammatory signaling contributing to systemic inflammation in disease. Moreover, patients with co-morbidities continue to pose a further challenge, stressing the ever-persisting need for alternative avenues when standard therapies are unsuitable or inadequate. This is where targeted therapies to the atheroma using

nanoparticles (NPs), for example, hold such promise, both as a way of minimizing adverse systemic side effects and enhancing target specificity. This is since NPs enable explicit control of drug activity (both spatial and temporal) and can also provide a more convenient means of administration [81]. Convenient and effective therapies with ease of administration may also help maintain patient compliance and achieve cost effectiveness. Alternative methods of drug delivery such as this are hence pertinent in alleviating, at least in part, pressure on healthcare systems, especially when treatment requires regular and continuous administration over a long period (which is typical in atherosclerosis). Although there are still limitations and drawbacks preventing the use of many NPs in clinical practice [81], many are currently undergoing development due to the aforementioned benefits (see [82]).

6.3 Alternative avenues: nutraceuticals and anti-inflammatory cytokines?

Beyond reducing levels of pro-inflammatory cytokines using monoclonal antibodies, other potential avenues include increasing the levels of anti-inflammatory cytokines (using recombinant forms) or cells that produce them (e.g. regulatory T cells), and even exploiting nutraceuticals to alleviate inflammatory burden. Nutraceuticals, food constituents with health benefits beyond nutritional value, represent promising alternative preventative/therapeutic avenues that can be combined with current pharmacological therapies for the treatment/prevention of CVD [5, 83]. Particular attention has been given to omega-3 polyunsaturated fatty acids (n-3 PUFAs) [84-86], green tea catechins [87-89] and olive oil polyphenols [90-92]. This is mainly due to their identified anti-inflammatory and antioxidant activities, with the potential of attenuating atherogenesis and disease progression at a cheaper cost in comparison to traditional pharmaceuticals. Recent results obtained from the REDUCE-IT (Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention) trial showed that daily intake of 4 g of icosapent ethyl (a purified eicosapentaenoic acid ethyl ester), by patients with established CVD/diabetes/other risk factors on statin therapy, significantly reduced incidence of ischemic events [93]. This demonstrates the potential of further inhibiting residual risk of MACE via statin and nutraceutical co-therapy. Advancements have also been made in the field of omega-6 PUFAs, with an example being dihomo-γ-linolenic acid (DGLA), which has demonstrated a range of anti-inflammatory/atherogenic actions *in vitro* [94], as well as the ability to attenuate atherogenesis and disease severity in *ApoE*$^{-/-}$ mice [95]. Potential anti-inflammatory cytokines worth mentioning with recent advancements made in knowledge of their signal transduction mechanisms are IL-33 [96] and transforming growth factor (TGF)-β [97], which are both considered anti-atherogenic. IL-33 can attenuate foam cell formation via upregulation of IL-10 expression [98] and TGF-β is released by anti-inflammatory, M2 macrophages, which possess atheroprotective effects and are associated with plaque regression [99]. Importantly, augmenting levels of either cytokine resulted in attenuated disease burden presented as smaller lesion size or less abundant foam cells in previous studies using *ApoE*$^{-/-}$ mice [100, 101].

7. Conclusion

Exciting advancements have been made as we progress into more streamlined therapeutic approaches using biomarkers to tailor treatments to individual patients (“personalized

medicine"). Successful translation of years of research to medical care is always momentous and even more so when the therapy yields success and patient benefit is observed. Whilst targeting inflammation has always been recognized as a potential therapeutic strategy for systemic conditions such as atherosclerosis, it was not until the last few years that this was applied to large clinical trials with successful results. Results of these trials emphasize the importance of identifying and characterizing key signaling pathways and the accompanying downstream targets implicated in systemic inflammation, which will no doubt fuel future research to develop the panel of biomarkers used for better assessment of patient status and suitability for treatment. More research focusing on developing cost-effective strategies that can be applied abundantly on the front line of medical care and how to mitigate the threat of compromising immunity is now required, in order to successfully reduce morbidity and mortality associated with atherosclerosis and other chronic inflammatory disorders.

- **Future perspective:**

We are progressing through an exciting new era of anti-atherogenic therapies, with results from recent clinical trials showing the promise and effectiveness of targeting IL-1β for the prevention of atherosclerosis-related events [35, 49, 63, 65]. This will no doubt fuel the exploration of other potential target cytokines, including IL-6, IL-18 and IFN-γ, which are other key pro-atherogenic cytokines closely involved with IL-1β. As the efficacy of anti-cytokine strategies is verified, focus will shift to identifying and developing novel, cost-effective agents and more convenient, targeted drug-delivery methods to enable wide-scale application of anti-cytokine therapies in front-line medical practice, given the high prevalence of atherosclerotic disease. In light of this, repurposing existing agents used to treat other systemic inflammatory disorders for the prevention of primary and secondary MACE is already being explored. An example of this being colchicine [56, 61, 62], a broad-spectrum anti-inflammatory that targets neutrophils. A number of phase 3 clinical trials are underway with an explosion of data expected upon their completion, guided by promising results yielded from the LoDoCo trial [56]. Nutraceuticals is another potential alternative avenue, with green tea catechins, olive oil polyphenols and omega-3 PUFAs all having anti-inflammatory (and other anti-atherogenic) activities [5, 83]. Promising results of the REDUCE-IT trial [93] emphasize the potential of co-therapy with existing pharmacological agents. In the next 5 to 10 years, we will likely see an increase in similar clinical trials that supplement different anti-inflammatory nutraceuticals as part of currently standard treatment strategies to enhance CVD prevention, along with continual development of alternative drug-delivery methods to enhance drug target specificity and minimize unwanted side effects.

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- **Executive Summary:**

Atherosclerosis

- Atherosclerosis is a chronic inflammatory disorder of the vasculature
- Accumulation of cholesterol within the arterial wall stimulates infiltration of immune cells to the area.
- Plaque formation and development involves various cell types and chronic inflammation.
- Eventual plaque rupture results in clinical manifestations, including myocardial infarction, cerebrovascular accident and peripheral vascular disease, usually as a result of thrombosis.

Inflammation in atherosclerosis

- Pro-inflammatory cytokines and chemokines are implicated in the entirety of the disease.
- In atherosclerosis, the balance of anti- and pro-inflammatory cytokines is tipped in favor of the latter, fueling the development of therapies aimed at resolving this chronic inflammation in order to improve outcomes.
- IL-1β is a potent, key pro-atherogenic/inflammatory cytokine whose production can be stimulated by multiple factors.

Clinical trials exploiting anti-inflammatory/cytokine therapies

- Results from the CANTOS trial show that targeting pro-inflammatory cytokine, IL-1β successfully reduces levels of IL-6 and CRP, lowering incidence of secondary cardiovascular events.
- Whilst the CIRT proved less successful, exploring the reasons behind this is important both in knowledge development and to guide future therapies.
- Another promising anti-cytokine/inflammatory agent is colchicine, which also successfully reduced occurrence of secondary events and is currently being exploited in a number of other clinical trials with more results due soon.

Other avenues

- Targeting other key, pro-inflammatory cytokines such as IL-6, IL-18 and IFN-γ are possibilities worth pursuing.
- Boosting levels of anti-inflammatory cytokines, such as TGF-β and IL-33, are also potential avenues that may dampen inflammation-driven processes.
- Nutraceuticals may be exploited for their anti-inflammatory effects as a cheaper, non-pharmacological alternative.
- There is a need to identify/develop suitable, cost-effective agents that can achieve this and be readily applied to medical practice.

- **Figure legends:**

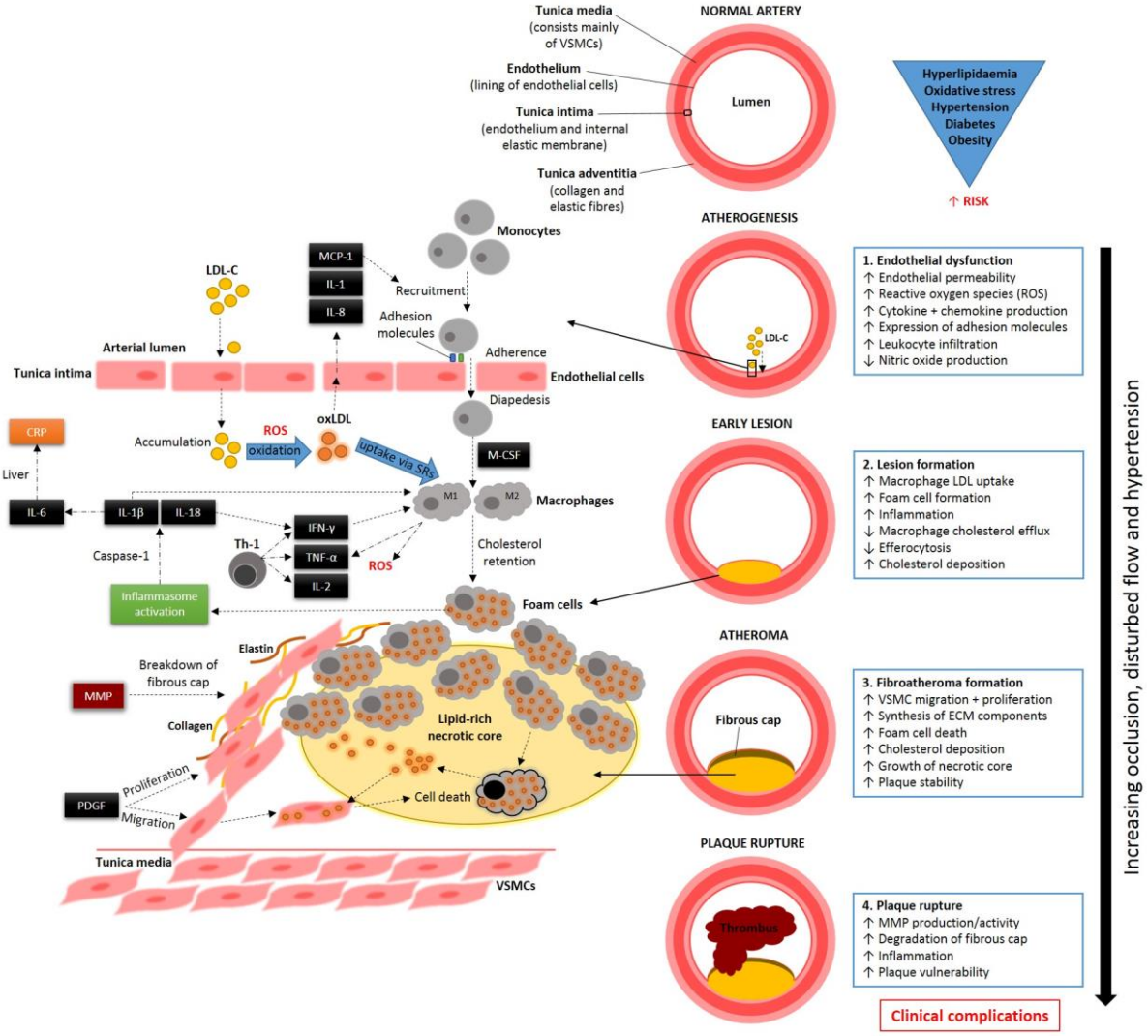

Figure 1. Inflammation in atherosclerosis. The main pro-atherogenic processes involving key cytokines are illustrated. Atherogenesis arises from passage of LDL through the endothelium and into the subendothelial space, where its modification, predominantly oxidation by ROS and other processes, into oxLDL triggers an immune response, resulting in infiltration of immune cells, particularly monocytes and T cells. Cholesterol crystallization within macrophages can trigger inflammasome activation, resulting in the production and release of potent pro-inflammatory cytokines. Plaque progression is facilitated by enhanced inflammation and an increasingly-necrotic phenotype. Plaque destabilization and rupture is facilitated by the breakdown of the fibrous cap. See text for more details. *Abbreviations*: MCP-1, monocyte chemoattractant/chemotactic protein-1; IL, interleukin; CRP, C-reactive protein; IFN, interferon; TNF, tumor necrosis factor; Th-1, T-helper cell; PDGF, platelet-derived growth factor.

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- **Reference annotations**: authors should highlight 6–8 references that are of particular significance to the subject under discussion as **"* of interest"** or **"** of considerable interest"**, and provide a brief (1–2 line) synopsis.

3. Ramji DP, Davies TS. Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev.* 26(6), 673-685 (2015). **[* Describes in detail implication of cytokines in atherosclerosis]**
4. Buckley ML, Ramji DP. The influence of dysfunctional signaling and lipid homeostasis in mediating the inflammatory responses during atherosclerosis. *Biochim. Biophys. Acta.* 1852(7), 1498-1510 (2015). **[* Describes in detail how pro-inflammatory cytokines are involved in dysregulation of lipid homeostasis]**
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49. Ridker PM, Everett BM, Thuren T *et al*. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* 377(12), 1119-1131 (2017). **[** Details the first successful large clinical trial using a monoclonal antibody against IL-1β]**
73. Libby P. Interleukin-1 beta as a target for atherosclerosis therapy: biological basis of CANTOS and beyond. *J. Am. Coll. Cardiol.* 70(18), 2278-2289 (2017). **[** Describes the rationale for targeting IL-1β for the prevention of cardiovascular events]**